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We have conducted an analysis of 19 cell lines derived from human breast cancers with the goal of identifying potential novel genomic amplicons connected with the molecular pathogenesis of this disease. We employed the Restriction Landmark Genomic Scanning method for this. No evidence of novel amplicons was detected in this limited sample of specimens. Possible reasons for these results are discussed.

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FOREWORD

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(5) INTRODUCTION

Several distinct types of genetic alterations are believed to be causal in the formation and progression of tumors (1, 2). Gene amplification is a prominent molecular lesion in a variety of human tumors, including breast cancers (1-8). By increasing the copy number of a gene through amplification, high level expression of the corresponding gene product may result; where the gene is a classical dominant oncogene that positively regulates cell growth, pathological dysregulation of proliferation that contributes to malignancy can ensue (1, 2). Gene amplification *per se* may also be a reflection of a state of "genomic instability" characteristic of neoplastic cells (4).

Amplification of several growth control-associated genes in carcinomas of the breast has been observed at various frequencies (reviewed in (3, 8)); these include the growth factor receptor genes ERBB2, EGFR, IGF-IR, and FGF receptors 1, 2 and 4; the proto-oncogenes CMYC and CYCD/ BCL1/ PRAD1; and the p53-binding protein MDM2. [Although amplification of the genes for the growth factors FGF3 and 4 has also been observed in breast carcinomas, these are not thought to be significant in pathogenesis; instead, these are believed to reflect passive amplification of the genes "driven" by the adjacent CYCD locus (see (8))].

Gene amplification in tumors has been detected most frequently by the "shotgun" approach of DNA blot hybridization to a battery of cloned oncogene probes, sometimes guided by prior biochemical or immunoassay data. This approach suffers the drawback that amplification of previously unknown sequences can not be detected in this way. Rarely, amplified novel oncogenes have been discovered when present in tumors in characteristic cytogenetic structures (9, 10). In general, however, identification of novel amplified sequences in tumors has been problematic.

A rather recently described technique, comparative genomic hybridization (CGH), has offered a new approach to detecting genetic amplification (and deletion) in tumors (11). Briefly, this is a two-color fluorescence *in situ* hybridization (FISH) method that allows relative gene dosages to be assessed on a genome-wide basis; detection is based on the relative kinetics of reannealing of differently-colored tumor-derived and normal cell-derived fluorescent DNA probes to normal metaphase spreads. A relative excess or deficiency of a particular gene sequences in a tumor results in a brighter tumor-specific or normal cell-specific signal at the corresponding chromosomal locations on the metaphases, respectively. Preliminary CGH studies of breast cancer specimens have demonstrated candidate novel (distinct from ones noted above) genomic amplifications (12, 13, 14). <u>CGH does not permit direct isolation of genomic sequences that have been found to be amplified</u>.

(6) BODY

I. Experimental Strategy and Methods

Restriction landmark genomic scanning (RLGS; "genomic scanning" for short) is a genetic analysis tool developed by Hayashizaki and coworkers (5, 6, 15, 16). In RLGS, mammalian (or in some instances, yeast) DNA is first cut using a restriction enzyme (such as Not I or Asc I) of the special class that cuts relatively infrequently (i.e., ~10,000 times in the 3 billion basepair haploid human genome); the ends so generated are specifically radiolabeled using a DNA polymerase to fill-in the ends, and the reaction is heat-killed. The DNA is then

cut with a second enzyme, such as Eco RVor PvuII, that cuts quite frequently. The cut-and-labeled DNA is next resolved according to molecular length for the first time using agarose tube gel electrophoresis. A third restriction enzyme (such as Hinf I or PstI) is then diffused into the agarose tube gel in order to cut the DNA a third time *in situ*. The DNA is next resolved by length a second time using a polyacrylamide slab gel. The end-radiolabeled DNA is visualized by autoradiographic exposure of X-ray film. A highly reproducible pattern of "spots" is observed which is characteristic of the species under study and the combination of restriction enzymes employed; each spot corresponds to one genomic location. Typically ~2,000 spots are well-resolved when the gels are cut into easily handled pieces; this corresponds to a sampling of the genome at *ca.* 1 megabase pair intervals on average. The intensity of each spot is proportional to the number of gene copies present in the cell of the corresponding genomic region.

The majority of spots in human DNA are 2-copy, because two identical alleles are present at the genomic locus of origin (7). A few highly reproducible spots in normal mammalian DNA are of greater than two copy intensity ; detailed characterization of these by sequence database analysis and cloning has shown that they represent primarily ribosomal RNA genes (17). About 10% of human spots are 1-copy due to the presence of polymorphic alleles (e.g., each allele makes a separate 1-copy spot) (7, 18). It has been found that RLGS can be a useful tool for the identification of genetic amplification and deletion events associated with tumorigenesis (19, 20). Comparison of normal and tumor DNA from neuroblastoma patients, for example, has revealed amplification of the N-myc gene and of an additional novel locus in two patients not having N-myc amplification (19). Genetic deletion events, possibly corresponding to tumor suppressor gene deletions, are commonly observed in tumor DNAs (15, 19).

II. Results and Discussion

A. RLGS analysis of human breast carcinomas

We had proposed to use RLGS as an approach to 1. identify, and 2. clone, candidate novel genomic amplifications in human breast carcinoma (HBC); the ultimate goal of the proposed studies was to isolate new dominant oncogenes significant in the pathogenesis of HBC. To this end, in year 1 of support we surveyed fifteen HBC cell lines and primary tumor specimens derived at the University of Michigan Health System, as detailed in the proposal and statement of work; these specimens had been pre-screened by Southern blotting to exclude the "major" amplicons in HBC (see above). In year 2 we analyzed a further four cell lines that had shown evidence of novel chromosome 8 amplification via CGH. <u>We have not found convincing RLGS evidence for novel amplicons in any of these materials</u>. This result is consistent with similar studies that we have conducted on human malignant gliomas (Radany, et al., unpublished results) in which a candidate novel amplification (as opposed to previously characterized ones) was detected in only one of 23 specimens that had not been pre-screened by CGH (see below). We did find evidence of an unusual apparent demethylation (19) of two of the dominant ribosomal RNA gene-associated spots (17) in three of the initial HBC specimens. The significance of this result is uncertain; demethylation of repetitive elements in tumors, detected by RLGS, has been reported previously (19). Since this finding is unrelated to the goal of detecting novel amplicons in HBC, it is not being pursued at this time.

We can offer several reasonable, and non-mutually exclusive, explanations for the failure to find novel amplifications in the specimens examined to date:

- The initial 15 specimens simply did not contain any amplicons.

  In the case of the cell lines studied, this could possibly be a function of the biology of those tumors that are able to be established in culture.

- Novel amplicons most frequently co-exist with known ones (like ERBB2) and so any such specimens would have been excluded in the initial group of tumors by the Southern blot pre-screening.

  Note that tumors that acquired the ability to readily amplify genomic loci could tend to do this for multiple loci (most often including the ones that are common along with any novel ones). In this case, amplification of novel loci could be regarded as a nonrandom event that is selected against by the pre-selection approach we employed. Note, for expample, that two of the lines characterized by CGH as displaying chromosome 8 amplification also had amplification of ERBB2 on chromosome 17q, and thus would not have been assayed in the initial group of tumors.

- Genomic amplification in human breast carcinomas frequently entails increased methylation of NotI sites within the amplicons associated with genes that do not drive the amplification.

  Such a situation could exist if genes whose products are negative regulators of growth were clustered near the genomic positions of dominant oncogenes. One may readily envision in this case that prior loss of transcription (as by methylation of the respective promoter elements) of these former hypothetical genes would be a mechanistic requirement for the evolution of the genomic amplification. Since methylated NotI sites can not be detected by RLGS, the effective size of such amplicons would be very small, and so RLGS might not afford sufficient detection sensitivity.

- Amplicon-derived spots may have been missed using enzyme combinations for which we have little experience in the analysis of normal DNA, and for which paired tumor line plus normal tissue specimens were not available.

  We utilized a number of unusual enzyme combinations for the intensive analysis of the four lines having CGH evidence of chromosome 8 amplification in an effort to maximize the probability of detecting any spots from this region. As matched normal tissue is not available for these lines, we were obliged to characterize the normal spot patterns for these combinations using normal human lymphoblastoid lines as the source for the DNA. It is possible that multicopy spots in the normal DNA represented uncommon polymorphisms of the multicopy ribosomal DNA clusters, while spots in closely similar locations on the gels were in fact tumor amplicon-derived.

Alternatively, the chromosome 8 region in question may have very few NotI sites that can be resolved on the usual gels.

- For reasons that are not understood, the chromosome 8 CGH results for the four lines analyzed in the second group were incorrect.

  That is to say, the signal in this region of the genome that was interpreted as representing amplification may have been an artifact of some process, such as an unusual density of some repetitive element in that region, say that caused a spurious increase in hybridization of one of the FISH probes.

B. An improved "spot" cloning method

While it has proved possible to directly clone amplified DNA sequences out of material extracted from excised gel "spots" in several cases, using conventional plasmid vectors and electroporation-competent *E. coli* cells (19), we have not been uniformly successful in this approach. As the goal of Technical Objective 2 is to clone the DNA sequences corresponding to spots that derive from candidate novel amplicons in HBC, during Year 1 we experimented with alternative cloning approaches that might be more consistently successful; we focused on a PCR-based strategy

We found that it is feasible in some cases to combine an affinity purification step for the initial NotI-ended genomic fragments with the ligation of a specific oligonucleotide "tag" (the so-called SP6 promoter universal primer). This tag made it possible to extensively purify, subsequently, a NotI genomic fragment of interest isolated from a gel spot and thereby remove the large excess of HinfI-HinfI fragments that are present. This step is done following ligation of a Hinf I-ended amplimer oligonucleotide. In this way, a final PCR amplification step using SP6 and the Hinf I amplimers yields a product that can be efficiently cloned or [in the case of spots from gels of yeast artificial chromosome (YAC)- containing yeast hosts] sequenced directly.

With greater experience we have come to appreciate limitations of this approach. We have found that many spots can not in fact be amplified successfully in the second step of this method. We speculate that this may be due to the high GC content expected of the CpG islands from which many of the RLGS arise and/or secondary structures in these sequences. Admixture of organic cosolvents in the amplification reaction did little to remediate this problem.

C. Combined RLGS genomic analysis using tumor and YAC DNAs

The resources of the Human Genome Project are of potential value in the proposed experiments. We investigated the feasibility of combining YAC contigs and the corresponding physical and genetic map information with analysis of tumor amplicons using RLGS. In a pilot study, amplification of the EGFR locus in human tumors was found to give rise to a constellation of high intensity spots We demonstrated that these spots could be ordered on

the human physical and genetic maps by RLGS analysis of several YACS from the human chromosome 7p12 region.

Dr. Samir Hanash of this institution, who is a collaborator with us on RLGS studies unrelated to the goals of this grant, has found evidence of another novel amplicon in HBC on chromosome 17 q in the vicinity of the gene TBX2 (21)(see Figure 1). YACs are available for this region (21). In the light of our developed expertise in RLGS analysis of YACs, we analyzed this contig in an effort to derive probes from this region as well (e.g., Figure 2). This excercise gave us additional experience with the analysis of YACs with a variety of enzymes including AscI as the first cut one. However, we did not identify any additional YAC-derived spots for the TBX2 region in addition to the two shown in the figures using other such enzymes. Thus, this contig analysis was of no help in localizing the "driving" gene for amplification near TBX2.

(7) CONCLUSIONS

We have not succeeded in isolating molecular probes for candidate novel amplicons in human breast carcinomas using RLGS. However, we have come to believe that RLGS is best used in conjunction with CGH pre-screening of tumor specimens to find ones that contain novel amplifications. Such tumors may, in turn, be studied with many combinations of restriction enzymes for the RLGS to identify a maximal number of amplicon-derived spots; the latter may then be cloned to derive amplicon probes. We were able to implement the latter strategy for only four cell lines, because relatively few CGH studies have been reported for lines, as opposed to primary tumor specimens, for which unlimited DNA is not available. The CGH-informed studies here were targeted at the region 8q specifically (that is, the four lines were selected on the basis of having this finding). The failure to identify spots derived from this region may be a specific property of it, and thus the promise of the RLGS-CGH approach for analysis of other genomic regions

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LIST OF APPENDICIES

Appendix 1--Figure Legends and Figures

Figure Legends

Figure 1. Novel 17q amplicon near TBX2 in a human breast tumor. Three novel high intensity "spots" are indicated as arrowheads. One of these was cloned and sequenced, and found to originate from the TBX2 locus (see text). The other two candidate amplicon derived spots have proved refractory to cloning.

Figure 2. RLGS analysis of YACs from the vicinity of TBX2. Two "spots" corresponding to ones visualized in the gel of a human breast tumor RLGS (Figure 1) are indicated.

FIGURE 1

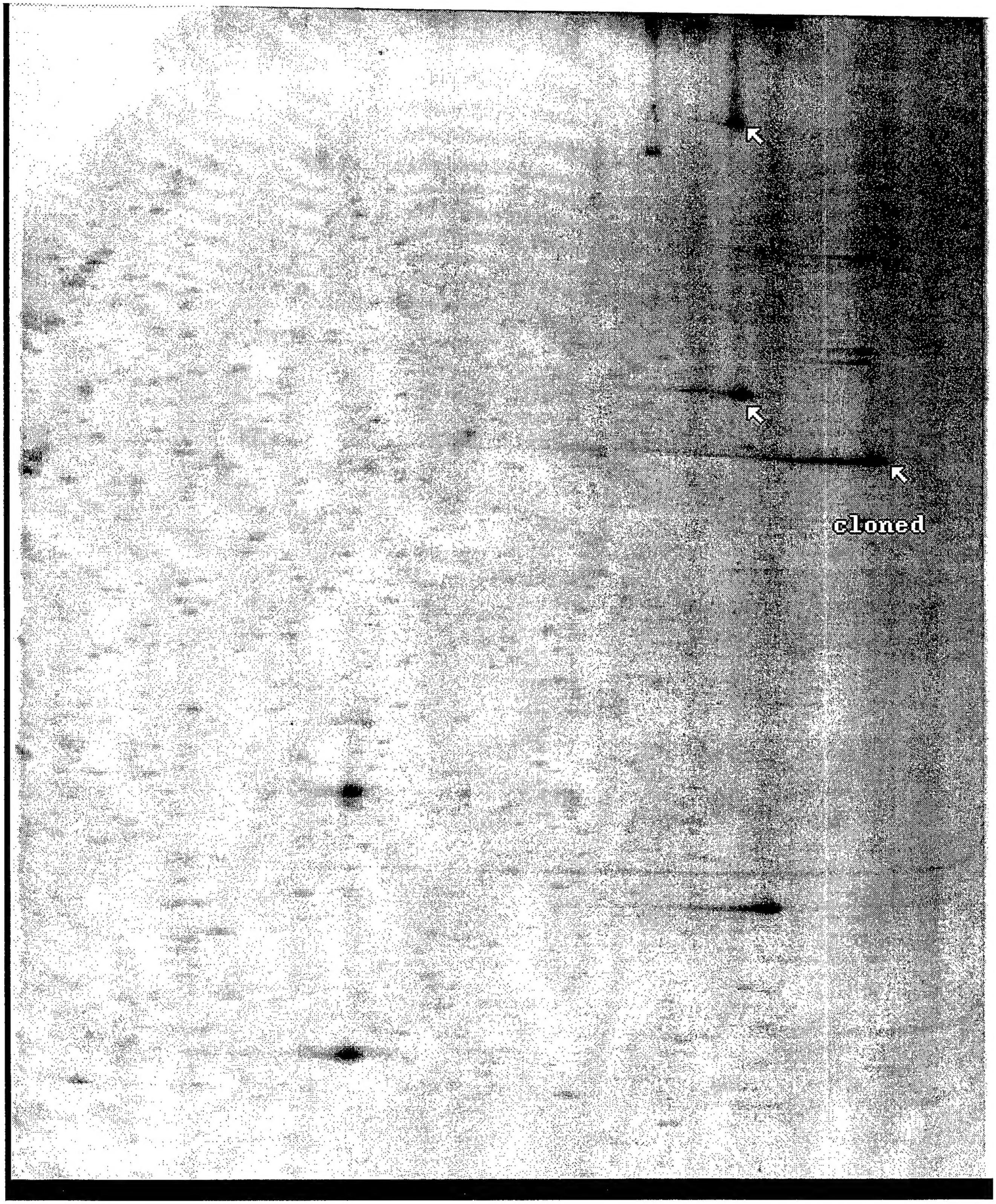

FIGURE 2